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Genetic Evidence for Coinfection of Honey Bees by Acute Bee Paralysis and Kashmir Bee Viruses[1]

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Nucleotide sequence analyses were used to identify acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) isolated from a single honey bee colony. Most of the bees in this colony carried KBV. Some individual bees also carried ABPV, a coexistence not yet seen between these two viruses. Implications of coinfection on viral efficacy are discussed, along with a restriction enzyme assay that can be used to discriminate between these two widespread viruses. © 2001 Elsevier Science (USA)

***Key Words: Apis mellifera;* acute bee paralysis virus; Kashmir bee virus; coinfection; nucleotide sequence; picorna-like virus.**

INTRODUCTION

At least 16 viruses, primarily members of the picorna-like group of RNA viruses, are found in honey bees (Bailey and Ball, 1991). Two widespread viruses in this group, acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV), have been identified in Europe, Australasia, and North America (Allen and Ball, 1996; Hung *et al.*, 1996a,b,c). Both the prevalence and the importance of these 2 viruses remain poorly understood. ABPV originally was identified as a laboratory phenomenon in England in 1963, where it had never been associated with disease or mortality of bees (Anderson and Gibbs, 1988; Bailey, 1965; Bailey *et al.*, 1963). Nevertheless, ABPV since has been implicated in the mortality of honey bees from colonies infested with the parasitic mite *Varroa jacobsoni* (Ball, 1985). ABPV also appears to be common as a sublethal infection in apparently normal bees from Canada and Italy (Bailey, 1965).

KBV was first isolated from samples of the Asian honey bee, *Apis cerana*, leading Bailey and Woods (1977) to propose an Asian origin for this virus. Further surveys, however, have identified variants of this virus in European honey bees, *A. mellifera*, from Canada, India, Spain, Australia, and New Zealand (Allen and Ball, 1995; Anderson and Gibbs, 1988), Fiji (Anderson, 1990), and the United States (Bruce *et al.* 1995; Hung *et al.* 1995). Thus, it is impossible at this stage to infer either the geographical or the host origins of this virus. The effects of KBV on honey bee health in the field are equally unclear (e.g., Andersen, 1991). Hung *et al.* (1996a) used immunodiffusion techniques to identify KBV in dead and morbid honey bees in an apiary in the United States, although they did not determine whether this virus was the actual cause of death.

The polymerase chain reaction (PCR) provides an opportunity to identify and characterize honey bee viruses, even when these viruses are at low levels (Hung and Shimanuki, 1999a, 2000; Grabensteiner *et al.*, 2001; Benjeddou *et al.*, 2001). Surveys using PCR can therefore be used to define the absolute geographic and host ranges of bee viruses. Perhaps more importantly, PCR can been used to screen bees for viruses nondestructively (Hung, 2000), an important feat for experimentation and for certifying that honey bee queens are free of viruses prior to importation. Here we use direct reverse-transcription PCR (RT-PCR) to identify ABPV and KBV from individual workers collected from a colony known to harbor both viruses (Hung *et al.*, 1996a). We present the first genetic evidence that honey bee viruses can infect the same honey bee simultaneously. Finally, we describe and test a restriction-enzyme assay that can distinguish between ABPV and KBV on the basis of RNA sequence differences in a region encoding the viral capsid protein. This assay allows the rapid determination of bees infected by ABPV or KBV or by both viruses.

[1] Nucleotide sequences for the capsid protein gene reported here have been registered with GenBank under Accession Nos. AF263723–36 and AF264688–92.

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MATERIALS AND METHODS

Twenty-three adult worker bees from honey bee colony BRL-9 in the Bee Research Lab apiary, Beltsville, Maryland were assayed for KBV and ABPV. In addition, 5 bees that had been inoculated with ABPV isolated from bees in Florida (kindly provided by B. Ball, IACR-Rothamsted) were analyzed. Virus RNA was amplified by direct RT-PCR following the protocol of Hung and Shimanuki (2000), with an annealing temperature of 50°C. Negative and positive controls were used for each RT-PCR run.

Two loci were amplified, one only for KBV and one for both viruses. Primers KBV1 (5′-GATGAACGTCGACCTATTGA-3′) and KBV2 (5′-TGTGGGTTGGCTATGAGTCA-3′) from Stoltz *et al.* (1995) amplified a 414-bp segment of the RNA-dependent RNA polymerase gene in KBV only. A second primer pair, CAPIS (5′-GGCGAGCCACTATGTGCTAT-3′) and CAPIA (5′-ATCTTCAGCCCACTT-3′), was designed from ABPV sequence data generated by Ghosh *et al.* (1999); (GenBank Accession No. AF126050) to amplify a 401-bp segment of the gene encoding the capsid protein of both ABPV and KBV. This section of the capsid shows strong sequence similarity with the VP3 section of the *Hepatitis A* viral genome.

The RT-PCR products were excised as single bands from 1.5% agarose gels after electrophoresis and were then purified with the Gel Eclipse DNA Purification Kit (Tetra Link International, Amherst, NY) or directly purified with the NucleoSpin Extraction Kit (Clontech Laboratories, Inc. Palo Alto, CA). Purified products were sequenced on an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA).

Sequence data for both the RNA polymerase and the capsid protein gene fragments were compiled and aligned using Omiga 2.0 (GCG-Oxford Molecular Products, Madison, WI). We included the ABPV capsid protein sequence generated by Ghosh and co-workers in the alignment, along with outgroup sequences from *Drosophila* C virus (Johnson and Christian, 1998) and cricket paralysis virus (King *et al.*, 1987), since these are the closest described relatives to these bee viruses (Evans and Hung, 2000).

Amino acid sequences for the bee viruses and cricket paralysis virus were inferred after alignment and comparison with *Drosophila* C virus. Aligned sequences were exported to the phylogenetic analysis software PAUP 4.03 (Sinauer Associates, Sunderland, MA). Phylogenetic relationships were inferred from 401 bp of aligned sequence from the capsid protein, using a heuristic (tree-branch-bisection) search algorithm. The robustness of these phylogenies was inferred by bootstrap replication (n = 10,000 replicates).

To confirm virus coinfection, and as a tool for future surveys, we developed a restriction-fragment assay to distinguish between ABPV and KBV. Restriction enzyme *Alu*I (recognition site = AGCT) cuts PCR products from both viruses at nucleotide position 43 of the 401-bp amplified product, but cuts only KBV at position 151 of the same product, generating a clearly distinct signal. In contrast, restriction enzyme *Bgl*II (restriction site AGATCT) cuts only ABPV (at position 183/401). In separate reactions, we digested 5 μl of each PCR product to completion according to conditions recommended by the supplier (Roche Biochemicals). The digested DNA was analyzed by agarose gel electrophoresis using a 2.0% (w/v) agarose gel stained with ethidium bromide. After destaining, DNA bands were visualized with a UV transilluminator and then photographed.

TABLE 1

PCR Identification Using the RNA-Dependent RNA Polymerase Gene (KBV Only) and the Capsid Protein (Both ABPV and KBV) in 28 Individual Bees

Sample ID	RNA polymerase	Capsid protein
BRL1	KBV	KBV
BRL2	KBV	ABPV
BRL3	KBV	KBV
BRL5	KBV	KBV
BRL6	KBV	KBV
BRL7	KBV	n/d
BRL10	KBV	n/d
BRL11	KBV	n/d
BRL12	KBV	KBV
BRL13	KBV	KBV
BRL14	KBV	KBV
BRL15	KBV	KBV
BRL17	KBV	KBV
BRL18	KBV	n/d
BRL19	KBV	ABPV
BRL20	KBV	Neg.
BRL21	KBV	ABPV
BRL22	KBV	ABPV
BRL23	KBV	ABPV
FABPV5	Neg.	ABPV
FABPV6	Neg.	ABPV
FABPV7	Neg.	ABPV
FABPV8	Neg.	ABPV
FABPV12	Neg.	ABPV

Note. Neg., negative in PCR amplification; n/d, no sequence data. Samples BRL1–23 are from Maryland. Gaps in numbering refer to the six samples that tested negative for both viruses. Samples FABPV5, 6, 7, 8, and 12 reflect viruses cultured from Florida bees by B. Ball.

RESULTS

Nineteen bees from colony BRL-9 tested positive for KBV using KBV-specific primers for the polymerase gene (Table 1). PCR products from 10 of these were sequenced and confirmed to be greater than 97% identical, within the range found across North American variants of KBV (Hung *et al.*, 2000). These sequences also were used to place the current samples within a

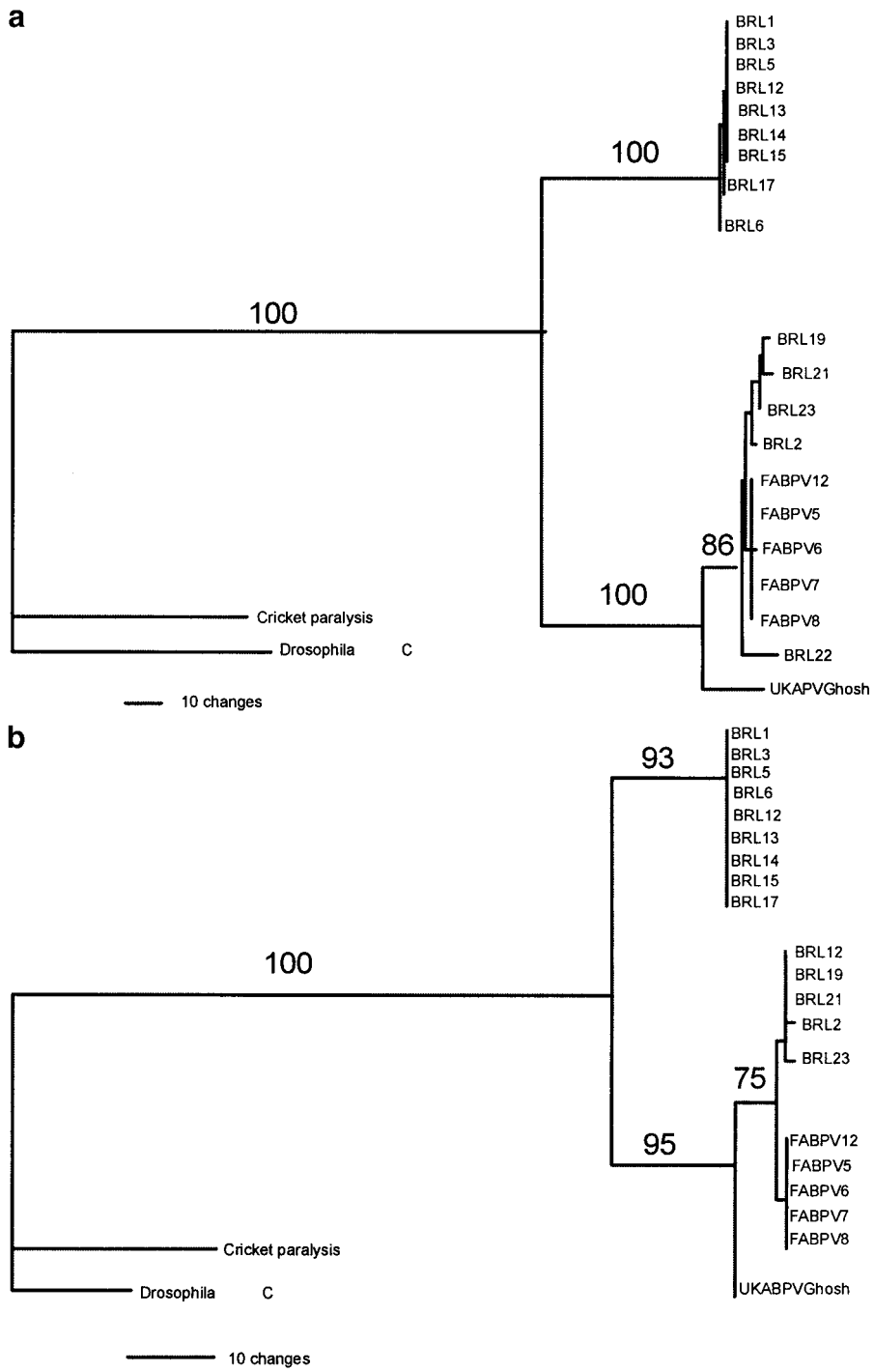

FIG. 1. Phylogenetic relationships across RNA virus capsid proteins based on (a) the RNA sequence and (b) the inferred amino acid sequence. Bootstrap values are shown above branches. Horizontal branch length is indicative of sequence changes.

described clade of KBV (e.g., Hung and Shimanuki, 1999b; GenBank Accession No. AF027125).

When 14 of the 19 KBV-positive samples were sequenced for the capsid protein gene region, 5 (BRL2, 19, 21, 22, and 23) showed an RNA sequence at the capsid protein gene that differed greatly from the remaining 9 samples (BRL1, 3, 5, 6, 12, 13, 14, 15, and 17; Table 1).

Figure 1a shows the phylogenetic relationships of these 14 samples, along with several confirmed ABPV samples and the two outgroup viruses (*Drosophila* C virus and cricket paralysis virus). Two distinct clades

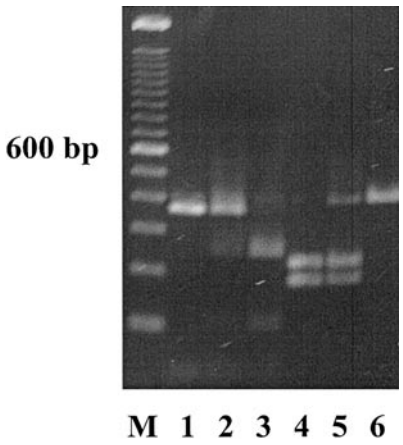

FIG. 2. Restriction fragment length polymorphism for the capsid protein gene. Patterns shown for samples infected by ABPV alone (FABPV7, lanes 1 and 4), ABPV and KBV (BRL2, lanes 2 and 5), and KBV alone (BRL1, lanes 3 and 6). The PCR products in lanes 2–4 were digested with *Alu*I and those in lanes 5–7 were digested with *Bgl*II. Lane 1 is a 100-bp DNA ladder.

were strongly supported by phylogenetic analyses (bootstrap values = 100%). The Florida ABPV samples (FABPV5–8, 12) and a subset of the samples from colony BRL-9 were paired with the European ABPV sequence (UKABPVGhosh). All of the North American ABPV sequences were more closely related to each other than to the ABPV sample from Europe (bootstrap value 96%; avg. similarity = 98–100% within the United States and 90–92% between the United States and the European samples). This ABPV clade differed from the second, putatively KBV, clade by 27% at the RNA level (range = 26–28%). The characterization of members of this second clade as KBV is supported by both their polymerase gene sequences (Table 1) and by immunological assays (not presented). There was very low genetic similarity at the RNA level in all of these bee viruses and their putative sister species, *Drosophila* C virus (40% sequence identity, range = 39–41%).

The phylogeny inferred from the amino acid sequences was identical to that found using the RNA sequences (Fig. 1b). Within the United States, all KBV isolates were identical at the amino acid level, while they were only 79% identical to members of the putative ABPV clade. Similarly, members of the ABPV clade from BRL-9 were 99–100% identical with each other, 97% identical with the ABPV samples from Florida, and 93% identical with the ABPV sample from Europe. As a class, the honey bee viruses differed from *Drosophila* C virus by approximately 55% (range = 53–60%) at the amino acid sequence level.

All five bees suspected of being coinfected with ABPV and KBV were clearly distinguishable using restriction-fragment analyses with *Alu*I and *Bgl*II (Fig. 2). These restriction analyses provide more convincing evidence for virus coinfection than do the sequence analyses alone, since they can identify coinfection even when the titers vary substantially between the two viruses (see lanes 3 and 6 in Fig. 2).

DISCUSSION

Our study presents strong evidence that ABPV and KBV can infect the same honey bee simultaneously. While virus coinfection is anticipated, evidence for coinfection by specific viruses is rare. Dall (1985) reported that there was no instance of mixed infection of KBV and sacbrood virus (SBV) in the pupae that they tested. However, Anderson and Gibbs (1988) reported simultaneous inapparent infections of KBV, SBV, and black queen cell virus (BQCV). When activated, KBV suppressed the replication of SBV and BQCV. In our study KBV levels appeared lower than those of ABPV in all five coinfected samples (see example in lanes 3 and 6 of Fig. 2). Quantitative RT-PCR can now be used to assess the frequency and direction of suppression between these two viruses.

The relative ranges of KBV and ABPV remain unresolved, as are any interactions between these two virus species and their honey bee hosts. According to Anderson (1991), KBV may compete with ABPV for the same "niche" in honey bees, but "the climatic and environmental conditions of certain countries favor the existence of one virus over the other." Nevertheless, characterization studies by Allen and Ball (1995) showed that several strains of KBV are serologically related to ABPV and that "the use of different names for these viruses may be inappropriate." Our sequence analyses suggest that KBV and ABPV are in fact distinct viruses and that these viruses can be inferred to be different species. More extensive surveys are ongoing to determine the frequency of coinfection, while controlled experiments are needed to determine the impact of ABPV, KBV, and a coinfection of each of these viruses on honey bee health. Such studies will further establish the roles of viruses in honey bee pathology and can be used to mitigate any adverse effects of these viruses. Thanks to the arrival of RNA sequence data for most of the important honey bee RNA viruses (e.g., Ghosh *et al.*, 1999; Evans and Hung, 2000) and the use of these sequences for precise identification (Grabensteiner *et al.*, 2001; Benjeddou *et al.*, 2001), PCR assays can now be used to determine the rates of coinfection by a range of honey bee viruses.

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